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Plant Pathogen Forensics: Capabilities, Needs, and Recommendations

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INTRODUCTION

A biological attack on U.S. crops, rangelands, or forests could reduce the yield and quality of agricultural products, erode consumer confidence, affect nutrition, adversely impact the nation's economic health and international markets, and cause harm to the environment (31, 105, 188, 189, 190). Preparedness for a crop bioterror event must begin with recognition of the threat, which must lead to the development of a plan to thwart, or to respond to and attribute, an attack. However, most U.S. crop producers, crop consultants, and agricultural scientists have not focused on the possibility of deliberate plant pathogen introduction but instead have directed their efforts toward the prevention of accidental or natural introductions and the development of cost-effective disease management strategies. Should these efforts fail, responses typically address rapid eradication and/or long-term disease management. In this article, we build a case for the integration of the traditional discipline of plant pathology and the specialized field of forensic science, envisioning the birth of the new field of plant pathogen forensics. We describe the potential threat to plant resources and then focus on one part of a strong national security plan: the development of a program in microbial forensics and criminal attribution that addresses crop and other plant resource targets (5). Toward this goal, we review currently available information, technologies, and resources. Although they were developed originally for plant health or economic applications, many may be utilized also for plant pathogen forensics. We present recommendations for the prioritization of activities, including critical research, development of new technologies and infrastructure, and allocation of the human and financial resources needed to ensure a strong capability in plant pathogen forensics.

Vulnerability of U.S. Crops, Rangelands, and Forests

Worldwide, losses for the eight major crops that comprise half of the global croplands were estimated at $300 billion from 1988 to 1990 (129). In the United States alone, plants are subject to attack by over 50,000 different pathogens, primarily fungi, viruses, bacteria, and nematodes (105, 106). For any given region and crop, producers may deal with up to 10 to 15 serious plant diseases that can cause severe economic repercussions (137). About 65% of U.S. crop losses are due to nonindigenous (introduced) pathogens, amounting to an estimated cost of $137 billion annually (136). All crop pests (pathogens, arthropods, and weeds) combined cause preharvest losses of 42% and an additional 10% loss after harvest. Of these, 13% are due to plant pathogens, 15% to arthropods, and 13% to weeds.

Recent acute, but unintentional, introductions of nonindigenous plant pathogens (or their vectors) demonstrate the range of damage and consequences associated with newly introduced exotic pathogens (Table 1). If such an outbreak were to be caused by the intentional release of a naturally occurring or engineered biological agent, the political, economic, and societal impacts would be considerable.

In addition to natural outbreaks, crops are vulnerable to deliberate biological attack (5, 31, 105, 188, 190). Agriculture can be an attractive target for the introduction of pathogens as bioweapons because of its critical role in the infrastructure of most nations. In the United States, for example, agriculture and related industries comprise about one-sixth of the gross domestic product, or about $1 trillion annually. Moreover, 17% of U.S. employment is related to agriculture. Agricultural products comprise a major component of U.S. exports. Crops, rangelands, and forests occupy vast areas in the United States, covering half a billion hectares. Because regular surveillance of such extensive areas is not feasible, long lag times (months or even years) may pass between the introduction of a threatening pathogen and its detection, even if symptoms and signs are present. For example, an outbreak of citrus canker in Florida was first diagnosed in 1995 (164), but the causal bacterium was probably introduced 2 years prior to its detection. Similarly, *Plum pox virus* (PPV) was first detected in Pennsylvania in 1999 (99) but is now thought to have been introduced as much as 6 to 8 years earlier. Many of our valuable crop and forest species, bred for consistent quality and high yield, are planted as monocultures. Low genetic diversity increases the potential for pathogen spread and widespread damage. Finally, plant pathogens are generally easy to obtain, increase, transport, and deploy. Most pose little if any threat to the health of their human handlers, and the ethical barriers to deployment of a plant pathogen may be lower than those for human or animal pathogens.

In wealthy countries, a deliberate pathogen introduction event could (i) result in severe negative impacts on crop yield and quality, (ii) cause significant public shock and/or panic due to a loss of confidence in a portion of the food supply, and (iii) negatively impact the national economy, particularly in rural and agricultural sectors, due to yield decreases, reduced income from commodities sold, and the potential effects of quarantines and/or the loss of international markets. In countries of the third world, particularly those in which a single crop (such as rice or cassava) is the primary food commodity for a large segment of the population, it could lead to human hunger, suffering and political disruption, as have resulted in the past from natural and accidental plant pathogen introductions.

Plant pathogens of high risk for the United States that are designated select agents under the Code of Federal Regulations, title 7, part 331, by the Agricultural Bioterrorism Protection Act of 2002 and the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) (http://www.apsnet.org/online/feature/BioSecurity/ and http://www.cdc.gov/od/sap/docs/salist.pdf), are listed in Table 2. This list is parallel to select agent lists for human and zoonotic diseases, except that all plant pathogen select agents are nonindigenous pathogens not yet known to occur in the United States (termed "exotic" in the Plant Pest Act). Strict regulations, registrations, restrictions, and security are required for their handling and investigation (http://www.aphis.usda.gov/programs/ag_selectagent/index.html). Such regulations may be useful in attributing a crime involving a select agent. However, a plant pathogen select agent introduced into the United States with little likelihood of eradication may be delisted to facilitate the research needed for effective postintroduction disease management. The recent removal of soybean rust from the APHIS select agent list after the natural introduction of the causal fungus into the United States in the fall of 2004 (161, 171) and the concomitant delisting of PPV are two examples of this policy.

TABLE 1. Recent acute, but unintentional, examples of the arrival in the United States of nonindigenous plant pathogens and their consequences

Disease	Pathogen	Situation and consequences
Asiatic citrus canker	*Xanthomonas axonopodis* pv. citri (bacterium)	Florida's $1.4 billion citrus industry is severely impacted by bacterial canker, a disease that has arrived and been eradicated several times in the past. Eradication, the only effective management strategy known, includes both trees showing symptoms and adjacent, symptomless trees likely to harbor the pathogen. The recent eradication program cost more than $200 million and destroyed over 10 million trees (17). After some home and business owners sued to save their trees, control procedures were suspended and the disease continued to spread until the court decided in favor of the eradication strategy (67). The hurricanes that struck Florida in 2004 and 2005 caused significant pathogen spread beyond the previously defined eradication zones. As a result, USDA-APHIS has determined that the pathogen cannot be eradicated and is now developing a disease management strategy.
Bacterial wilt	*Ralstonia solanacearum* race 3, biovar 2 (bacterium)	This race of the wilt bacterium was inadvertently introduced at least twice in recent years into the United States via infected geraniums shipped from Africa and Central America to nurseries (90, 191). "Suspect" geraniums were quarantined and eradicated, and several nursery growers were forced out of business. However, the management policies addressed the possibility of cross-species transmission from infected geraniums to potatoes, a scenario feared by potato growers who face "zero tolerance" for the pathogen in shipments to foreign markets.
Sudden oak death (SOD)	*Phytophthora ramorum* (oomycete)	First detected in 1995 in Pacific coastline oak forests, this disease, caused by a fungus-like oomycete, now covers over 1,000,000 ha and has killed tens of thousands of trees in California and Oregon (64). The U.S. Forest Service has declared large regions of the Eastern United States to be high-risk areas (181). SOD threatens severe losses to the nursery industry, as many ornamentals could be quarantined or eradicated. California-grown camellias, azaleas, and rhododendrons are already quarantined by other states, and 59 other plant species, including rose, huckleberry, honeysuckle, lilac, and bay, are susceptible and at risk (177).
Pierce's disease of grapevine	*Xylella fastidiosa* (bacterium)	Pierce's disease threatens California's $2.8 billion wine, table, and raisin grape industries, which occupy 300,000 ha (143). The causal bacterium, disseminated by an unaggressive insect vector, the blue-green sharpshooter, had been present, but insignificant, in California since the 1880s. In the early 2000s, however, the arrival of a more aggressive vector species, the glassy-winged sharpshooter, substantially increased the impact of the disease, disseminating it rapidly. The disease continues to cause alarming losses.
Karnal bunt of wheat	*Tilletia indica* (fungus)	This fungal disease was first detected in the United States in 1996 in durum wheat in Arizona and California (196). Although Karnal bunt causes only minor reductions in wheat yield and quality, stringent phytosanitary trade restrictions have led to strict quarantines for wheat produced in zones in which Karnal bunt occurs. The resulting economic impact on rural communities is just as damaging as if the wheat had actually been destroyed. The restrictions have caused significant economic losses for some Texas and Arizona farmers (50), showing that in some cases the greatest damage from a plant disease may result from the negative impacts on the markets and the consequent loss of income to local producers (115).
Asian soybean rust	*Phakopsora pachyrhizi* (fungus)	Farm value of U.S. soybean production in 2003–2004 was $18.0 billion (USDA Economic Research Service) (http://www.ers.usda.gov/Briefing/SoybeansOilCrops/). In Asia, where this rust disease is endemic, annual yield losses reach 10–30% and losses in individual soybean fields reach 90%. A 30% yield loss in the United States would cost about $4 billion. Within the last decade, soybean rust moved from Asia to Africa and then to South America. It was first detected in the United States (Louisiana) in the fall of 2004 (161) and has since been found in several southern states (165). Rust spores likely entered the United States as a result of Hurricane Ivan (171). Arriving near the end of the growing season, the pathogen had little impact on U.S. soybean production in 2004. However, the fungus is now thought to be established on alternative hosts, such as kudzu, in warmer regions and may spread annually from these overwintering sources (138). Losses to U.S. producers and consumers could average between $240 million and $2.4 billion per year in the next 3–5 years. This case is an example of how advance anticipation of a probable introduction facilitated preparedness, including surveillance and enhanced diagnostics for early pathogen detection, the development of advisories and rapid response cascades, advance emergency registration of fungicides as a short-term mitigation, and the initiation of research for development of long-term strategies.

TABLE 2. Exotic plant diseases and plant pathogens on the U.S. Department of Agriculture's Animal and Plant Health Inspection Service regulated pathogens list (Code of Federal Regulations, title 7, part 331)

Disease	Pathogen[a]
Fungal diseases	
Asian soybean rust	*Phakopsora pachyrhizi**
Philippine downy mildew of corn	*Peronosclerospora sacchari*
Potato wart	*Synchytrium endobioticum*
Brown stripe downy mildew	*Sclerophthora rayssiae*
Bacterial diseases	
Citrus variegated chlorosis	*Xylella fastidiosa* (CVC strain)
Rice bacterial leaf streak	*Xanthomonas oryzae* pv. oryzicola
Citrus greening	*Liberobacter africanus*, *Liberobacter asiaticum*
Bacterial wilt	*Ralstonia solanacearum* race 3, biovar 2
Viral diseases	
Plum pox virus disease (of plums, peaches, and other stone fruits)	*Plum pox virus**

[a] *, delisted in 2005.

Although no endemic (or indigenous) plant pathogens are on the select agent list, many pose threats to agriculture that are equal in severity to those on the list. Further, many of the endemic pathogens are readily accessible and could be used in an illicit manner against crops. Since criminal penalties may now be imposed for unlawful possession or use of listed (as well as illicit use of unlisted) pathogens, a sound and reliable forensic analysis system is needed for purposes of attribution.

History of Plant Pathogens as Bioweapons

Biological warfare against agricultural targets is not a new idea. Over many decades, various state-sponsored research programs have been established with the goal of using weaponized microorganisms as part of the country's military arsenal (31, 32, 105, 188, 190). The United States conducted research involving a number of pathogenic microbes, including the biological agents causing anthrax, foot and mouth disease, and rice blast. Germany had programs during both the First and Second World Wars, whereas the former Soviet Union conducted programs from the Second World War through the Cold War, as did Iraq, beginning with the Iran-Iraq War. Other countries are thought to have had biological weapon programs aimed at agriculture and food production. Evidence found in caves in Afghanistan suggested interest by Islamic militants in the weaponization of the fungus that causes wheat rust. Other countries that have explored microbes as potential weapons include Canada, France, Japan, and the United Kingdom. Agricultural bioterrorism may be an outgrowth of such thinking and activities.

There have been no documented cases, as yet, of the deliberate use of pathogens to attack crops or other plants. However, a posture of preparedness dictates that reasonable steps be taken to ensure that appropriate crop biosecurity capabilities be in place before a devastating event occurs, not afterwards. To that end, the examination of natural or accidental plant pathogen or pest introductions can provide insight into the possible impacts of a successful deliberate attack. For example, the potato blight epidemic in Ireland (1845 to 1846) led to extensive famine, resulting in the deaths of 1 million and the emigration of an additional 1.5 million Irish (29, 96). Brown spot of rice contributed to the Great Bengal Famine of 1943. In the United State, a leaf blight in 1970 destroyed about 20% of a corn crop valued at $1 billion (151).

ROLE OF MICROBIAL FORENSICS IN CROP BIOSECURITY

Whether plant pathogens (or their products, e.g., toxins) are used deliberately as weapons to cause social or economic damage or are introduced inadvertently into a new area, it is important to determine the source, the method, and the time of the introduction, as well as those responsible for it (21, 23, 24, 41, 86, 88, 122, 154). Analyses related to such determinations fall into both the forensic sciences and epidemiology. Forensic science is the application of scientific methods in the investigation of possible violations of the law, where scientific knowledge and technology provide evidence in both criminal and civil matters. The discipline provides specific support for investigative and law enforcement efforts in which the ultimate goal is attribution, the determination of the perpetrator of a criminal act (18, 20, 21, 22, 23, 24, 25). Forensic analyses include the use of tools for sampling, packaging, shipping, storage, microbial identification and discrimination, chemical analyses, epidemiological modeling, bioinformatics, and other considerations. Because the safety and confidence of the public must be preserved, and because the legal ramifications of criminal attribution and prosecution require unusual investigative validation and stringency, a forensic investigation involving a plant pathogen may require use of methodologies that address different questions and/or have higher resolution than those normally used in disease diagnosis and plant pathogen identification. Forensic application requires the confidence necessary for ultimate attribution (19, 20).

The degree of confidence with which forensic analyses can support identification of a specific microbe, reconstruction of its method of introduction into a particular location, and identification of the perpetrator depends on many factors. Lag times may occur at several stages of the investigation. The first is the time between the introduction of a pathogen and its detection, which is affected by many factors, including weather conditions before, during, and after the introduction. A second lag is the time required to develop and execute an appropriate sampling protocol. Protocols must include validated techniques that minimize the time between on-site sample collection and arrival at a forensics laboratory. Third, the time required for stringent laboratory assessment and the resolution, reliability, and repeatability of the chosen analytical methods affects the success of a forensic investigation. In many cases it may be far easier to determine exclusion (assurance that a particular pathogen or person is not involved in the incident) than absolute attribution (evidence that may uniquely associate a particular pathogen/isolate or person to the incident).

A critical first question with respect to a plant disease outbreak is whether a crime has occurred. Many diseases are already endemic, and once-exotic diseases that are not eradicated may become endemic relatively quickly after introduction. An intentional introduction of a plant pathogen as a

TABLE 3. Proposed plant pathosystems developed for the assessment of current microbial forensics capabilities, gaps, and needs

Pathogen	Pathogen type	Disease	Vector/transmission
Soybean mosaic virus	Virus	Soybean mosaic	Aphids, seed
Tomato spotted wilt virus	Virus	Tomato spotted wilt	Thrips
Plum pox virus	Virus	Plum pox (Sharka)	Aphids, cuttings, grafting
P. syringae pv. tomato	Bacterium	Bacterial speck of tomato, pepper, and edible brassica	Wind-blown rain; infested soil
C. michiganensis subsp. *sepedonicus*	Bacterium	Ring rot of potato	Infested tubers
U. maydis	Fungus	Smut of maize and teosinte	Wind blown

biocrime or bioterrorist event might not be recognized as such. Thus, tools for better pathogen resolution, more-relevant background information, and more-robust surveillance mechanisms are needed to better evaluate whether a disease is natural or human incited. On the positive side, crop producers and plant pathologists are already poised to move quickly to apply management strategies to control disease. Therefore, rapid determination of whether criminal activity has occurred is crucial so that responders know if the event should be handled as a crime, with appropriate steps for attribution, or solely as a containment effort. Forensic science can assist in this endeavor.

As the nascent discipline of plant pathogen forensics develops, standard crime scene processing and evidence handling protocols must be validated and adapted to plant pathogen forensics applications. It may be appropriate to develop some new technologies specific for crime scenes involving crops, forests, nurseries, orchards, or rangelands. A thorough analysis is required to identify and assess the information, capabilities, tools, and resources already in existence. Once these are brought to bear on the new applications of forensic science, it will be possible to identify remaining gaps and the needed capabilities to fill them, a step that will serve as the basis for the development of a forward strategy. This subdiscipline of forensics specifically targeted toward microbial pathogens, as applied to bioterrorism and biocrimes involving humans and animals, has been developing over the past few years. However, few if any field or laboratory methods, standard operating procedures (SOPs), or protocols have yet been specifically developed and rigorously validated for application to plant pathogens. As plant pathogen forensics becomes established as a separate subdiscipline of forensic science, a major early area of opportunity will be to critically assess, select, and shepherd existing methods, SOPs, and protocols through an appropriate process so that "sets" of validated "tools" are available and defensible should a crop bioterrorism event occur. To accomplish these near- and long-term goals, plant pathologists and forensic scientists (especially those working in microbial forensics) need to plan and work together.

USE OF SURROGATE PATHOSYSTEMS AS MODELS

In this article we explore forensics with a focus on plant pathogens. Since it would be impossible to address all plants and pathogens of interest, we have focused, where possible, on several specific model pathosystems (systems inclusive of the pathogen, the plant host, the insect vector [if applicable], and the environment) (Table 3) that, in future research initiatives, can be exploited easily outside of biocontainment facilities. In some cases, where information and/or examples are lacking among these model pathosystems, we cite examples from other plant pathogen-host systems. Although our research focuses on case studies from a U.S. perspective, these principles and concepts apply globally.

Five of the six model pathosystems are damaging pathogen-host-environment relationships that are currently established in the United States, where they cause substantial losses to the nation's agricultural productivity. High-caliber research programs are already in place, and a significant body of knowledge has been accumulated for each pathosystem. These pathosystems were selected to represent unique features of critical importance to forensic science so that research on the models will address real issues and answer real problems. The long-term utility of these model pathosystems (and of others not described herein) will be in the application of general principles, established from rigorous research, to any pathogen that might be deliberately introduced. To address the possible challenges of such a transfer of principles, our sixth model, PPV, was chosen to represent a USDA-APHIS-listed select agent. Although PPV was recently removed from the select agent list, this model pathosystem highlights the differences, both beneficial and restrictive, of working on an exotic and regulated pathogen. Our sample pathosystem models provide insight for the near- and long-term research investments required to achieve a strong and effective U.S. program in plant pathogen forensics.

COMPONENTS OF A STRONG MICROBIAL FORENSICS CAPABILITY

The application of forensic methods to a plant disease outbreak requires specific steps in forensic microbiology [F. W. Nutter, Jr., abstract from the Am. Phytopathol. Soc. Annu. Meet., Phytopathology **94**(Suppl.)**:**S77, 2004], including (i) careful documentation of disease characteristics; (ii) sampling the potential crime scene; (iii) identification of the pathogen to the race, strain, isolate, or isolate mixture; (iv) selection of appropriate mitigation response measures; (v) further characterization of the pathogen to identify likely sources; and (vi) attribution or exclusion of pathogens. Mistakes made during early stages of this process, i.e., during disease characterization and sampling, cannot be corrected at later stages of the investigation.

Sampling Methods, Sample Size, and Quality

On-site disease assessment. Initial assessment of the disease in the field should be done prior to any disturbance of the site.

This analysis should include the pattern of disease occurrence and any other field characteristics deemed relevant or unusual. Certain disease incidence patterns may be typical (or atypical) of a natural event [Nutter, Phytopathology **94**(Suppl.)**:**S130, 2004]. If possible, aerial and satellite images should be obtained prior to any ground disturbance. On the ground, areas with symptomatic plants should be delimited by application of global positioning system (GPS) technology prior to commencing with detailed sampling in affected areas.

SOPs for the collection of microbial forensic field samples must allow for variation among crop species and suspected pathogens. Forensic field samples may include whole plants, selected plant parts, plant surface swabs or exudates, soil (with or without root tissue), suspected insect vectors, natural or irrigation water in or near the fields, air samples, and/or biological samples (alternative weed hosts and soil or aquatic organisms, etc.). Containers must be clean and unused, and the samples must be collected directly into the container. Minimum documentation includes an administrative log, a sample log, the complete chain of custody, a collection site map(s) sufficient to allow repeat sampling from the same location (within or among fields), and a laboratory submission or transferal document providing detailed information on the crop, field history, and environment. Photographs showing symptoms, field layout, and other relevant details may supplement, but not replace, this documentation.

Characteristics of a good sample. What constitutes a "good" sample varies depending on the patterns of disease intensity, the pathogen, and the host. If multiple disease foci are present in the field, samples should be collected from a representative number of these locations, as well as from outside the focal areas [Nutter, Phytopathology **94**(Suppl.)**:**S130, 2004]. Since pathogen titers often differ in leaves, stems, roots, and flowers and with the distance from the site(s) of initial infection, it is important to sample from different plants and different plant parts. The ease of pathogen collection may vary with the season; for example, tree fruit phytoplasmas overwinter in tree roots and move into above-ground branches in the spring, while PPV is absent from tree samples collected in summer when the temperature rises above 30°C. When pathogen detection is carried out with sensitive assays such as enzyme-linked immunosorbent assay (ELISA) or PCR, it is often practical to combine tissue samples from several plants and analyze them together as a pooled sample; this form of group testing allows a larger proportion of the plant population to be tested with a minimal number of assays, thereby improving the detection limit (77) and reducing laboratory resource demands. A drawback of sample pooling is the loss of spatial and disease incidence information that would result from individual sample processing; however, plants from positive pooled samples could be retested individually in a second round of assays if information on the exact location and disease intensity of each infected plant is needed.

If necrotic lesions are present, it is best to sample from the lesion edges, where living plant tissue better supports active pathogen growth, as the lesion centers may subsequently be invaded by saprophytic microbes. Seeds are a good source of seed-borne pathogens such as *Soybean mosaic virus* (SMV) (74), and underground stems and tubers may serve as a source of pathogens, as occurs with the potato ring rot bacterium, *Clavibacter michiganensis* subsp. *sepedonicus* (43). Certain specialized pathogen structures, such as the galls of the corn smut fungus, *Ustilago maydis*, or the tumors produced by the crown gall bacterium, *Agrobacterium tumefaciens*, may be collected directly (38, 54).

Sample size and sampling pattern. The number of samples collected should be representative of the impacted area and be defensible scientifically and legally. Sampling pattern and sampling size considerations have been described for numerous pathosystems, including those that involve exotic or once-exotic pathogens such as PPV (78) or the citrus canker pathogen, *Xanthomonas axonopodis* pv. citri (117). In general, sampling for detection (i.e., presence or absence of a pathogen or disease) in a given field requires a different sampling pattern and sample size than sampling to determine the disease incidence or severity in the field. Sometimes, presence-absence data at the field level (prevalence) can be more important for forensic purposes than incidence or severity data, e.g., to determine the overall geographical extent of the disease or in deciding whether a given field should be placed under quarantine. In such cases, sampling can concentrate on high-risk areas within a field, such as borders or wet areas, depending on the pathogen.

In most forensic applications, detailed information on disease incidence or severity will be needed to develop spatial disease intensity maps to identify potential points of inoculation. In this case, sample size and sampling pattern considerations are critical. For assessment of disease incidence, Delp et al. (47) advocate the use of a stratified random sampling pattern in which the field is first divided into several strata (e.g., regions of higher or lower disease risk), followed by the random collection of samples within each stratum. Using this sampling design, percent error in disease estimates is reduced considerably compared with commonly used systematic sampling designs such as diagonal or W-shaped patterns. Among the systematic sampling designs, entire-field X- and W-shaped patterns are equivalent to each other and superior to diagonal or partial-field sampling patterns (101). When applying these sampling designs in the field, the sampler must be mindful of the fact that there may not be a single point of inoculation. For example, deliberate release of a pathogen by airplane may result in line or area sources of inoculum (128).

Logistics. Collection and documentation of 30 to 40 samples could occupy a two-person team for 8 to 12 h, particularly if travel is required between sample locations. Protocols for forensic field sampling should be designed in consideration of the number of available personnel; similarly, sample numbers should be reasonable in the context of the analytic and volume capabilities of the facility performing the laboratory analyses and/or diagnoses. However, no protocol should be designed solely based on resource limitations. The use of custody seals will alert the recipient to any tampering between the time of collection and receipt of the samples.

Because it is not possible to imagine every possible scenario that may require a microbial forensic investigation, a general SOP may not always be available. This limitation should not preclude attempts to collect critical evidence; however, the bases for current protocols and the investigator's experience should be relied on when adapting existing procedures to unique situations. When applying this "common sense and

experience rule," all steps and information accrued must be well documented. When samples are obtained from multiple locations (fields and locations within a field, etc.) appropriate decontamination of personnel and equipment is necessary to prevent the investigator from becoming a vector, spreading the pathogen, contaminating pathogen-free samples, and possibly resulting in false positives. The extent of decontamination will depend on the pathogenicity, virulence, aggressiveness, survival, and mode(s) of dissemination of the suspect organism.

Sample storage. Long-term storage of forensic microbial samples prior to analysis may be necessary; thus, great care must be taken to preserve the integrity and security of the samples. Some plant pathogens may be stored more successfully than others. Storage of viable pathogen cultures is a matter very different from preservation of desiccated leaf tissue, seeds, or fruits. Documentation of environmental conditions during storage is required, and chain-of-custody records must reflect all aspects of storage conditions and exposure to the environment, including records of individuals who may have access to the samples.

First detectors and first responders. "First detectors" on the scene of a deliberate plant pathogen introduction are likely to be growers, crop consultants, Master Gardeners, extension agents, or other local personnel not affiliated with the government. "First responders," individuals authorized to respond and take action after a potential deliberate introduction, generally arrive on the scene later, after being notified by first detectors. Note that the designation "first responders" differs here from that traditionally used for human targets, where the first responders are police or firefighters, etc. Clearly, timely and effective management of a crime scene will be impossible unless first detectors and first responders are equipped with the knowledge and skills to recognize that a crime has occurred and to react appropriately. Although ongoing efforts by the National Plant Diagnostic Network (NPDN) (http://www.npdn.org/) include training of first detectors and first responders (170), the number of plant pathologists trained in field applications of the discipline is on the decline. If the current levels of funding for extension, applied research, and plant disease epidemiology continue to decline, this lack of personnel will become one of the most serious gaps in crop biosecurity efforts.

Epidemiological Tools and Models To Support Forensic Analysis

The close relationships between public health and veterinary science with respect to epidemiological and forensic investigations are well recognized, as both seek to determine the origin and source of an outbreak even though subsequent response pathways may differ. Many parallels exist also between these two disciplines and that of plant pathogen forensics. The abilities to trace plant pathogens and to attribute their source(s) require analyses at levels from the whole plant to the landscape. Critical issues include (i) detection and quantification of the organism within the plant, field, or landscape; (ii) estimates of the initial infection time, based on anticipated environmental conditions and host growth stage; and (iii) the likelihood of spread to adjacent crops based on environmental conditions and/or vector activity. Such analyses require the use of epidemiological tools. Two broad epidemiological approaches that can support forensic analyses can be distinguished. Proactive approaches help investigators to understand variations in the intensity or geographical extent of a disease against a natural background, while reconstructive methodologies enable understanding of an event and facilitate establishment of the temporal sequence that comprised, as well as resulted from, it. The modeling tools used with both approaches are largely identical.

Climate matching, one of the most commonly used proactive epidemiological tools, helps to identify areas where and when anomalous disease events might occur, the probability that a pathogen could become established at a specific location, and how rapidly it might spread. Use of this tool has increased in popularity as global climate and species occurrence databases improve and expand (135). An empirical "bioclimate envelope" of the pathogen's environmental requirements is derived based on its current distribution, and long-term climate databases are then used to identify which geographical locations meet these requirements (8). Popular software tools include Climex (172, 173, 174), FloraMap (82), and AWhere-ACT (S. N. Collis and J. D. Corbett, Abstr. 4th Int. Conf. Integrating GIS Environ. Modeling [GIS/EM4]: Problems, Prospects and Research Needs, 2 to 8 September 2000, Banff, Alberta, Canada, http://www.colorado.edu/research/cires/banff/pubpapers/152/; F. Zermoglio, J. Corbett, and S. Collis, Abstr. New Tools Spatial Data Anal.: Proc. Center Spatially Integrated Social Sci. Specialist Meet., 10 to 11 May 2002, Santa Barbara, Calif., http://www.csiss.org/events/meetings/spatial-tools/papers/zermoglio.pdf). Bioclimate envelope analyses are often complicated by the existence of nonclimatic barriers to establishment and spread (155; M. J. Samways, Letter, J. Biogeogr. **30:**817, 2003), e.g., the absence of efficient vectors or susceptible hosts. Nonetheless, they are useful for first-pass analyses, especially for organisms for which more mechanistic models are not available (172). Recently, Pivonia and Yang (138) used the Climex system to assess the potential year-round establishment in North America of Asiatic soybean rust, an exotic fungal disease that was detected for the first time in soybean production areas in the southern United States in the fall of 2004 (161, 171).

When some of the environmental requirements of a pathogen have been determined experimentally, weather-based disease models can help define its likely locations of establishment and persistence (176, 194). This approach is used by the North Carolina State University-APHIS Plant Pest Forecasting System (http://www.nappfast.org/index.htm), a web-based modeling system that incorporates meteorological and crop distribution databases into a geographical information system (GIS). The system contains modules for several well-studied pest species, templates for new pests, and a generic infection model for exotic fungal plant pathogens (109). Another example of a generic weather-based model that can be adapted easily to a wide range of pathogens is the DYMEX simulator developed in Australia (172). When applied in a retrospective manner, disease models can determine whether conditions at a suspected release site were favorable, at a given time, for infection and disease development. Such analysis provides indirect evidence for whether intentional pathogen release at that site may have occurred (128).

Trajectory analysis utilizes complex atmospheric models and tracks airborne pathogen propagules in real time or in forecast

mode. Air parcels pick up spores in source areas and move them into upper-air streams, from which they are eventually deposited at distant locations where the likelihood of infection depends on the presence of a susceptible plant and a favorable environment. Atmospheric dispersion models such as the HYSPLIT4 (for "Hybrid Single-Particle Lagrangian Integrated Trajectory") model are used to calculate the most likely trajectories (28). Use of trajectory analysis in proactive mode is illustrated by the disease warning system for tobacco blue mold, which predicts the seasonal movement of the oomycete pathogen *Peronospora tabacina* from the Caribbean Basin and the southern United States northward along the east coast of the United States (110). The technique can be applied retrospectively to identify the likely source of an outbreak. For example, retrospective analyses of transatlantic wind patterns strongly suggested that the fungi causing both sugar cane rust and coffee rust were introduced into the Americas by aerial long-distance dispersal, the former from Cameroon in 1978 and the latter from Angola in 1970 (13, 16, 144).

Spatial disease data and associated infrastructure (GIS, GPS, and various remote-sensing platforms) have been applied in plant pathology for some time (107, 126, 140), and similar tools are now being developed and implemented by the NPDN to monitor and map outbreaks of agricultural threat organisms. A three-tiered approach to such analyses [Nutter, Phytopathology **94**(Suppl.)**:**S130, 2004] consists of (i) the acquisition of aerial and satellite images prior to conducting disease assessments on the ground; (ii) the ground-based assessment of disease incidence and severity in the affected area, in which the spatial pattern of disease is referenced by GPS; and (iii) the integration, mapping, and spatial analysis of remotely sensed and ground-based data in a GIS. In some cases, it may be possible to develop algorithms that can distinguish between natural and intentionally induced disease outbreaks based on the spatial pattern of disease. However, if an endemic pathogen is introduced at a single point and time, information on spatial patterns alone will add little to distinguishing between the two release scenarios. In such cases, genetic and population genetic analyses, as discussed below, will be critical for attribution.

Spatially interpolated high-resolution weather data and forecasts are based on simulations with a mesoscale weather model that ingests continental and global real-time atmospheric data, along with static information such as terrain and land use, to produce a numerical simulation with an output grid spacing of between 10 and 40 km (108, 152, 153); post-processing of the output interpolates data to a resolution of 1 km^2, allowing it to be linked to disease models to provide high-resolution information about future, present, or past disease risk (166) and providing an informational framework within which the previous or potential spread of an intentionally introduced pathogen may be estimated.

Critical forensic evidence related to time of infection can be provided by host and pathogen phenology data. Crop phenology data, derived from ground surveys, remote sensing (149), or crop models (121), can provide critical forensic evidence related to the time of infection, especially for pathogens that require defined host phenology stages for infection and/or with hosts that show age-related susceptibility variation (e.g., see reference 56). Information on pathogen phenology can be equally important, especially for determining time of infection. For example, information about leaf age and position, and lesion size and development, was used to determine lesion age in the search for the likely source tree of the current citrus canker epidemic in southern Florida (164). Time of infection can be reconstructed from propagule monitoring in relation to pathogen phenology. Spore samplers (61) can provide a continuous record of pathogen presence or absence in an area, especially when state-of-the-art high-throughput samplers are coupled with sensitive and specific detection procedures (e.g., PCR-based analyses or biosensors) (186). Such monitoring networks are currently being implemented for early detection of human pathogens in real time (e.g., the BioWatch program, the Biological Aerosol Sentry and Information System, and the Autonomous Pathogen Detection System) (58) and could be extended to include plant pathogenic threat organisms. If properly archived and documented, the samples collected routinely by such networks could be very useful for applications in microbial forensics.

Comparison and Validation of Current Microbial Forensic Identification and Typing Methods

When a disease outbreak occurs in a crop, the first question from a forensics viewpoint is whether it is a natural event or the result of direct human action, whether accidental or intentional. To answer this question with a high degree of confidence, one needs to establish whether the plant pathogen was absent from the location before the outbreak or was present but undetected until conditions became conducive for infection and disease development. Of course, proving the negative (or absence) is difficult or impossible. Where a pathogen is present, molecular typing must be carried out on multiple isolates, sufficient to distinguish with confidence all variants present from similar strains occurring naturally at that location or elsewhere in the world. Thus, high-level molecular typing for attribution is at the heart of microbial forensics. It is not yet available for all plant pathogens.

Continuum of attribution. Comparative interpretation of data from an evidence sample and a reference sample is a routine feature of a microbial forensic analysis. Three general categories of interpretation are "inclusion," "exclusion," and "inconclusive." The first two, inclusion (i.e., possibly originating from the same source or sharing a recent common ancestor) and exclusion (i.e., could not have come from the same source), are the two endpoints of a continuum of certainty with respect to attribution, while the variety of possibilities between the endpoints represent various degrees of inclusion and inconclusive data sets. Inclusion is achieved when the patterns or profiles generated from two or more samples are sufficiently similar that the samples could have originated from the same source. The measure of similarity should take into account all variation present in both samples. Because of the clonal nature of many microbial pathogens, it may never be possible to absolutely identify the source of the evidence. In some scenarios it may be possible to state only that two samples are similar or are more similar to each other than to other samples. An alternate definition of inclusion is a failure to exclude the possibility that the two samples had a common origin (or ancestry) or that they belong to the same group. An exclusion

event occurs when the sample patterns or profiles are sufficiently dissimilar that the two samples could not have originated from the same source (or are related too distantly). Lastly, an inconclusive interpretation is rendered when the data are insufficient to provide a conclusive interpretation (23).

International marketing requirements for agricultural commodities such as seeds or planting stock often require certification that one or more pathogens are, to a specified degree of confidence, absent. The high level of diagnostic accuracy needed for such assurances also may be applicable to microbial forensics. For example, the North American potato industry imposes rigorous testing for the potentially devastating ring rot bacterium, *C. michiganensis* subsp. *sepedonicus* (45). Zero tolerance trade restrictions for this pathogen have led to a major research focus in several countries to develop new detection methods that surpass current tests for sensitivity, specificity, and efficiency. Such newly developed methods may be the only diagnostic tests available for obtaining information to characterize the source.

Aside from such certification programs, the procedures typically applied to diagnose a naturally occurring plant disease for purposes of disease management are generally much less stringent, reliable, and reproducible than would be required for validated forensic identification. Regardless of the principle of the test, operators must appreciate the limitations of available assays to avoid overinterpretation and overrepresentation of results.

Criteria for selecting appropriate forensic typing methods. Critical characteristics of microbial typing for forensic applications include (i) universality, the ability to type all organisms within the taxon using a particular method; (ii) sensitivity, the percentage of actual positive samples detected (with no false negatives); (iii) specificity, the percentage of actual negative samples identified correctly (with no false positives); (iv) efficiency, the total percentage of correct test results; (v) reproducibility, the same result obtained consistently when a particular isolate is tested repeatedly; and (vi) resolution, the degree of attribution that can be obtained with a method.

For most plant pathogens, multiple methods of microbial identification and typing are available. Having results from multiple tests will increase the level of accuracy and confidence in microbial forensics investigations. Typing methods currently in use for plant pathogens include both nucleic acid-based and non-nucleic acid-based technologies.

Non-nucleic acid-based methods. The first and most important assays for both microbial forensics and management are those that determine the species of the pathogenic agent. A number of traditional methods, in use long before the advent of molecular biology, remain effective for some applications and may provide significant clues for pathogen identification in a forensics setting. Symptomatology, the ability to cause either no reaction or a "hypersensitive" (resistance) reaction on a nonhost plant; plant host range; insect vector specificity; and pathogen morphology (of bacterial colonies or cells, fungal colonies or fruiting bodies, or virus particles or inclusion bodies, etc.) are often the first steps in identification (90, 158).

The pathogen's host range and the host's specific response to the pathogen also are used for typing of plant pathogens. Species of many plant pathogenic bacteria are further divided into pathovars, based solely on the host range of the bacterium, and methods that define physiological processes or the complement of certain molecules are also used to define taxa. For example, BIOLOG (Hayward, Calif.) and other substrate utilization tests provide profiles of metabolic capabilities, while fatty acid methyl ester (FAME) analysis produces a profile of the microbe's fatty acid composition; in both cases the profiles of the test strain are compared with those in a database of species and strains for the closest match. The accuracy of such assays for microbial identification is limited by the population of characterized strains in the databases.

Like bacteria, plant virus strains belonging to the same species also may be discriminated by the comparative reactions of a set of plant species or cultivars within a species, known as differentials.

Fungi, because of their large genomes and complex life cycles, present particular typing challenges. For example, the fungal mating type, determined by plate mating assays, is the primary mode of identification of the model fungal plant pathogen *U. maydis* (142, 198). Although the mating assay is reliable and accurate, it is not very definitive for strain attribution because it does not measure other variations in the genome; on average about 1 of 36 of the cells will possess a given mating-type genotype in a random population. Thus, mating-type distinction is a good exclusionary tool but will not achieve absolute attribution.

Serological techniques. ELISA and indirect fluorescent antibody staining are serological assays commonly used for identification of plant pathogens, particularly viruses and bacteria. The sensitivity and specificity of serological assays vary with the titer and specificity of the antibody and whether the antibody is monoclonal or polyclonal (102, 180, 197). Recent adaptations by diagnostic industries for dipstick convenience and portability have enhanced the usefulness of these immunology-based technologies in the field. Cross-reactivity among closely related strains may be a problem; for example, the seven strains of SMV, identified on differential host cultivars (37), are chemically and serologically homogeneous at the coat protein level (74, 80).

Nucleic acid-based methods. The popularity of nucleic acid-based technologies has grown rapidly. Older methods, such as restriction fragment length polymorphism (RFLP), DNA fingerprinting, and phage typing are still valid and useful. However, complete genome sequences are now available for many economically important viruses and bacteria, and a few fungi, and others are in progress. Thus, many genetic markers are available for analyses. DNA probes, constructed for taxon-specific marker genes, are used widely. Sequencing of particular genome regions known to provide informative data, such as the 16S rRNA, the internal transcribed spacer region between the sequences coding for the16S and the 23S rRNA, or the *groE* or *recA* genes, is often used for bacterial identification. The sensitivity, specificity, and versatility of PCR have made it a method of choice for applications related to sequence analysis and comparison. PCR-based assays have been used widely, for example, in the typing of DNA viruses (100, 139). The discovery of repeated sequences in many bacterial genomes has given rise to a version called rep-PCR, in which electrophoretic banding patterns reflect different numbers and positions of repeated sequences (104, 145).

Real-time PCR has been used for genetic characterization of

bacteria (130, 157), viruses (112), and fungi (63). Although the technique is rapid and can be very specific, it may not be as sensitive as culture-based assays to detect pathogens present in plant extracts when PCR inhibitors are present, or with very small sample volumes, both of which can reduce sensitivity. For cultivable bacteria, PCR can be combined with isolation in BIO-PCR (160). In this assay, viable cells of the target bacterium are enriched in medium and thereby detected at extremely low original levels in seeds and other propagation materials. No DNA extraction is needed since the cells lyse during the initial denaturation step. For higher levels of specificity, BIO-PCR can be performed on membranes, although a possible disadvantage of membrane use is the chance for cross-contamination (159).

Multiplex PCR, in which primers against more than one target are combined in a single reaction mixture, can be employed to detect more than one species of bacterium or virus in the same sample (9). Assay and detection of multiple sites of a microorganism's genome can increase confidence in an identification. Such systems are currently in use for certifying vegetative plant propagules as virus free. Another PCR variant, reverse transcription-PCR (RT-PCR), is useful for plant viruses having RNA genomes. Reasonably "universal" primers have been developed for some virus families, genera, and "species" targeted to taxon-specific sequences (35, 91, 150). RT-PCR clearly differentiates between some strains of common viruses: for example, between PPV strains D and M (27) and SMV strains G2 and G7 (131), as well as between the common strain of *Potato virus Y*, PVY^O, and strain PVY^N (12). Kim et al. (92) conducted RFLP of RT-PCR products ("restrictotyping") to differentiate five Korean SMV strains. Many other new variations and assay combinations, such as multiplex PCR-ELISA and immunocapture PCR, have been developed (120).

The methods mentioned above are all limited by their reliance on a minute fraction of a taxon's many defining features. For nucleic acid-based methods, the accuracy of the comparison to some degree will be proportional to the length of the fragment (and/or the site) used in the analysis. Direct DNA-DNA hybridization methods provide information about the degree of similarity of entire genomes, without the need for actual sequence information.

Direct comparisons to rank the sensitivity and specificity of certain detection/diagnostic methods have been carried out for some plant pathogens (76, 85, 146). Many research programs shifted from traditional methods (symptomatology, electron microscopy, and host differential reactions) to PCR-based or immunological tests when the latter were demonstrated to be more sensitive or specific (12, 27, 112, 134). In limited cases, methods have been standardized among laboratories to ensure that comparisons between/among the groups were reliable (44). However, such test comparisons and standardizations are not frequently done, because the validation of methods at a level necessary for more rigorous challenge adds significant cost and generally is not required for managing a natural disease outbreak. Thus, for many diagnostic systems the relative effectiveness of one technology over another for critical identification is not known, and in fact, the "best" test will often depend on the "diagnostic" sites and methods available for a given taxon and the databases of information collected on the species and closely related strains and species. For example, in a recent analysis of multiple plant pathogenic strains of the ubiquitous bacterium *Serratia marcescens* (147, 199), different "identifications" were provided by BIOLOG, FAME, 16S rRNA and *groE* sequencing, and DNA-DNA hybridization because each of these tests measures or compares a different genome region, gene product, or phenotype. While exclusion and inclusion interpretations can be made with all of these methods, research is needed to establish the most reliable and informational methods for high-priority plant pathogens and to develop the reagents and databases for them.

The diversity of organisms within a microbial population must also be considered in evaluating typing methods. In a given plant, field, or region, some pathogen populations, such as those of *U. maydis*, are relatively homogeneous. For others, such as PPV, high population-level species diversity means that an individual sample from a single host will contain many mixed sequence variants (163). Researchers in the United States and the European Union are investigating the evolutionary tempo and drift of various regions within the PPV genome, and mutation "hot spots" within the genome could be targets for forensic analysis. Also relevant is the rate at which the pathogens change. For example, the genome of SMV appears to be more stable than that of PPV, although new strains of SMV are reported relatively frequently (49, 55, 92). Furthermore, some isolates described as new may actually be strains or recombinants of existing viruses (65). *Tomato spotted wilt virus* (TSWV) is quite variable because of reassortment of genomic segments and other mutations. For TSWV and related viruses, three loci (the N and NSm genes and the intergenic region) are used for comparisons. A rule of thumb is that isolates having $<90\%$ amino acid sequence identity in their N-protein sequences are distinct strains.

Importance of Genome Dynamics, Phylogenetics, and Systematics

Accurate identification of, and discrimination among, microbes is increasingly focused on their unique "molecular signatures" (41). Because microbial genomes have evolved in the past and continue to do so, genomic variability is an inherent characteristic of microbes. Pathogen populations vary in the rates at which they undergo genomic change and are subject to microevolutionary changes as influenced by the environment and their interactions with host species (either plant or insect vector), as well as with niche-neighboring microbial populations. All plant pathogens have coevolved to some extent with their plant hosts and, for those that are insect transmitted, with their vectors as well.

In considering the dynamics of bacterial genome change, it is useful to distinguish between a pathogen's core genome and its flexible genome (41). The core genome consists of genes ubiquitous in the bacterial species, encoding housekeeping proteins and other proteins essential for survival. These genes are less likely to undergo horizontal gene transfer and either evolve neutrally or are selectively constrained. The flexible genome consists of genes that vary among strains within a species, encoding proteins responsible for adaptation to a particular niche, host, or environment. Such genes, which may be associated with virulence, resistance to antibiotics or toxins, or the mobility of the genome or genome parts, evolve largely

through horizontal gene exchange (acquisition and loss). For viruses, regions of genes involved in host interaction or movement within the plant or by vectors, which may exhibit significant variability, would be useful for attribution.

Evaluation of genomic variability is a challenge to forensic investigation because of the difficulty in establishing tightly defined taxonomic groupings. However, certain aspects of variability among pathogen genomes can also provide outstanding support for identification. For example, a forensics-useful application became apparent when specific genomic regions of TSWV showed strong homology among strains from Florida and Georgia, and those strains could be resolved from strains from other parts of the world (133). Differences in fungal mating types may also be used as a forensic tool; *U. maydis* is a cosmopolitan species with much variation in mating type among populations, even within a small area (198). For some pathogens, specific regions of variability are potentially useful forensics tools. *Pseudomonas syringae* is a highly clonal and stable species, in which a genomic pathogenicity island (PAI) contains the "hypersensitive reaction and pathogenicity" (*hrp*) genes (1). The same PAI also contains the exchangeable effector locus (EEL), which is thought to have been acquired independently after the acquisition of the *hrp*-encoded PAI but before divergence of the pathovars (48). Divergence in EELs has occurred more recently through the acquisition of new effectors and by point mutation. Thus, the EEL may be useful in forensic investigations.

Pathogen populations are not homogeneous in nature. A given plant may be affected by a mixed pathogen population, including members of different pathogen kingdoms (e.g., viruses and bacteria in the same plant), different species (e.g., two phytoplasmas transmitted by the same vector species), or different strains/pathovars of a single species (e.g., pathovars tomato and maculicola of *P. syringae*). Even a natural population of a single strain of most pathogens may consist of many sequence variants. Most pathogen characterization is done, however, on very homogeneous populations initiated ("cloned") from single cells or propagules, a process considered essential for reproducible and comparable laboratory characterizations of microorganisms. Mutations of pathogens are common during laboratory maintenance and subculturing due to a lack of selection for characteristics needed to persist in nature. For example, pathogens propagated on artificial media without contact with host plant tissue may lose pathogenicity or aggressiveness after a number of passages, and insect-transmitted pathogens similarly may lose the ability to be so transmitted, even if propagated by grafting on a susceptible host plant. A pathogen stored frozen may undergo lower rates of mutation than one stored at higher temperatures or than pathogens in nature. Thus, there may be a degree of uncertainty regarding variation with those samples maintained in the laboratory. Comparative genomic sequence characterizations of mixed populations are needed to identify the degree of variation among individuals in a population, rates of mutation, and the extent of sequence divergence. Currently, such data are not available for most plant pathogens.

Populations of pathogens continue to undergo change in nature, although rates of change are not well characterized. In some cases, information may be gleaned from comparisons between populations in different countries or within regions of the same country. North American isolates of SMV were less diverse than those obtained from Asian countries, possibly because Asia is likely the center of viral origin and the opportunity for pathogen evolution has existed longer there (49). Deployment of host resistance genes in a crop species may select for variants that overcome the resistance; for TSWV and many other pathogens, this phenomenon can occur within only a few growing seasons.

The presence and number of extrachromosomal elements, plasmids, and viruses have been used for differentiation of cellular (nonvirus) pathogen strains. For example, the bacterium *P. syringae* pv. tomato DC3000 contains two plasmids, but although plasmids have been implicated in the pathogenicity of a number of plant pathogenic bacteria, curing experiments revealed no correlation between *P. syringae* pv. tomato plasmid presence and pathogenicity (26). Multiple prophages (virus sequences integrated into the genome) are present in many plant pathogenic bacteria, but their role in gene expression has not been well studied (26). All fungi have extrachromosomal DNA within their mitochondria and also may have linear and circular plasmids (68). For fungi that have no naturally occurring plasmids, such as the model fungus *U. maydis*, the presence of such elements in a field isolate of the fungus could be a sign of genetic modification by humans (179). Some fungi also have mycoviruses, which may reduce aggressiveness and affect gene expression (3, 116, 185).

Influence of Mutation, Evolution, and Environment

Molecular markers are important for microbe discrimination and forensics. Markers should be inherent, diagnostic, and Boolean. Inherent markers are those unlikely to change over the time since the suspect and the crime scene microbes separated. A change in state during this period reduces the strength of a possible association and identification. Inherent markers are less susceptible to modification by interaction with the environment. Examples of markers that depend on environmental and physiological conditions are protein modifications and distributions of expressed proteins and mRNAs. Genomic markers, such as the identity of bases and particular nucleotide positions, the presence or absence of specific nucleotide sequences, and the relative arrangement of nucleotide sequence stretches, are more likely to be inherent, although rare genomic features may be under such strong negative or positive selection imposed by environmental changes as not to be inherent. Markers that are selectively neutral relative to the microbe's environment allow application of standard population genetics theory for tracking through time and space. Some lack of inherency may be useful, however, when the property reflects a feature of the microbe's original environment. For example, a microbe's ^{18}O and ^{2}H isotope content can suggest in what water source it was produced (95). A diagnostic marker set discriminates among closely related pathogen strains, allowing confident exclusion of nonmatching suspects. The set will usually consist of several inherent markers rather than a single one, due to limitations on the confidence of discrimination associated with a single marker.

Simplistically, the probability of two microbes being derived recently from the same source, the attribution probability (p_a), is given by the formula $p_a = 1 - p_e$, where p_e is the exclusion

probability, the probability that they were not so derived. The desire in forensic microbiology is to have one of these probabilities be so high that there is little reason to doubt any associations or lack thereof.

DNA typing of humans is based on the frequency of alleles in the human population, the number of loci, and the mode of inheritance. The autosomal markers were chosen to be biologically (and thus statistically) independent. Independence is due to the presence of genes on multiple chromosomes and a high frequency of meiotic homologous recombination between distant markers on the same chromosome (except for those genetic markers residing on the nonrecombinant region of the Y chromosome or on the mitochondrial genome). As a result, the frequencies of the markers in the population can be used multiplicatively (with slight modifications) to calculate probabilities for exclusion or attribution. By a similar approach, most markers used with bacteria and viruses, and many fungi, are located on the same piece of DNA or RNA and thus may not be subject to frequent recombination. Exceptions are plasmids, horizontally transferred sequences, and genomes of multipartite viruses (69). Additionally, the degree of recombination varies among species. Because of the potential nonindependence of marker pairs, data are needed on both the frequencies of alleles at loci and the degree of linkage of the allele frequencies at pairs of loci to render the most effective estimate of the rarity of the nucleic acid profile.

Microbes evolve much faster than do humans or plants. Rapidly evolving regions of microbial genomes have promise for plant pathogen attribution-exclusion decisions because newly arising alleles may be novel and thus unique in the world population of that microbe. However, regions that evolve so rapidly that changes could occur during the time of divergence of the donor from that of the crime scene microbe lack the inherency mentioned above. They may still be useful, but the approaches for interpretation will be based more on a similarity/dissimilarity matrix. There is a need to identify the regions of plant pathogen genomes that are the most informative for the questions that may arise during a microbial forensics investigation.

Certain sites in genome sequences are under neither positive nor negative selection (94). These neutral sites should evolve at the same rates, since the processes that substitute one nucleotide for another in an organism are thought to be sequence independent and absence of directed selection is assumed. The frequency of differences at neutral sites of pairs of isolates with known divergence times can be used to calculate the mutation rates and the chance of multiple mutations at a particular locus. Knowing the neutral mutation rate and the frequency of differences at neutral sites between a suspect microbe and a crime scene microbe allows calculation of the time of divergence of the two microbes (under certain assumptions). If this time is within a window consistent with the suspected crime scenario, then a failure to exclude is supported. If the time scale is longer than the suspected separation of the two, exclusion is supported. In some microbial forensics cases the time may be a period of months, years, or decades. The confidence of attribution or exclusion based on a neutral site mutation rate depends on the accuracy with which that rate is known and the reliability of the observed difference frequency (and storage or environmental influences). Confidence in the neutral mutation rate increases with the number of isolate pairs used in its calculation. Thus, multiple sets of isolate pairs whose times since divergence from a common ancestor are known from historical records are needed. Sequences of regions containing multiple neutral sites, and improved computational methods for estimating the rates of change (59), also are important.

Although high confidence of strain identity is one goal of forensic attribution, strain identity alone does not always lead to absolute attribution. For example, the anthrax bacteria of the 2001 outbreaks were identified to strain, but identification of the perpetrator could not be ascertained directly from this information.

Background occurrence. Traditionally, when a plant pathogen is discovered in a geographic area in which the pathogen was previously unknown, a peer-reviewed note is published. Such information is often incomplete and nonuniform. The disappearance of a plant pathogen from an area is not frequently reported. Native plants and plants with unapparent symptoms are usually not surveyed, except when alternative hosts are being sought. It is common to provide some characterization with respect to pathogen markers, but marker characterization methods are not standardized. There is no single distribution source where such typing data are stored (87). However, some individual investigators or groups of investigators have created and are maintaining databases of isolates of concern to them. For example, there are extensive databases for the *Geminiviridae* (http://www.danforthcenter.org/iltab/Geminiviridae/ and http://gemini.biosci.arizona.edu/) and for two fungal genera, *Phytophthora* and *Fusarium*. At the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/entrez/), new sequence entries contain limited information about the host of isolation of the plant pathogen being sequenced and the rough geographical location. More-precise locations and dates of sampling and isolation are not usually provided.

Molecular markers for forensic analysis. As stated above, most plant pathogens lack a standard marker system. For those that do, markers are based on any of a variety of biomolecules and are taxon dependent. Rarely does a marker satisfy all three of the criteria defined above: inherent, diagnostic, and Boolean. Many marker systems were developed for use in systematics, the hierarchical assignment of organisms to a taxonomic classification. Several series of terms describe the hierarchy: strain, subspecies, species, genus, family, etc. There is a well-defined system for classification of viruses into families, genera, and species, but these taxa are being redefined with molecular techniques. Below the species level, further subdivision into subtypes, groups, or strains is common. Taxonomic levels do not reflect consistent evolutionary time periods. Although systematics has provided many useful markers, it does not always follow evolutionary descent patterns (187) and it is less relevant to microbial forensic considerations. Phylogenetics, in contrast, reconstructs the order of the organism's divergence from a common ancestor, so that under a certain set of assumptions, placement of a crime scene isolate on a phylogenetic tree identifies its closest known relatives or most recent common ancestor. Phylogenetic analyses, using a specific gene or genomic region, have been conducted on many plant pathogens. Meaningful trees require considerable sequence variation in the targeted portions of the genome, but such variability

should not interfere with reliable sequence alignment (119).

Few plant pathogen marker systems have been used widely enough to acquire good estimates of the frequencies of particular markers in populations. For most, the scope of the population is geographically restricted to a plot/field, county, state, or region, sometimes a nation. Seldom are data on worldwide frequencies calculable. Of marker types, nucleotide sequence is the best developed because more isolates have been examined by this method than by others. However, the diversity of microbes of potential phytopathogenic threat is so large that obtaining complete sequences of enough microbes for a good level of background knowledge is a daunting task (175). Genome microarrays and subtractive hybridization approaches may be good alternative technologies for resequencing (11). Indeed "high-resolution differentiation between closely related" microbes was obtained in a microarray study in which 295 of 300 pairs of bacterial strains were statistically differentiable (192).

Confidence levels. The overall mean mutation rate, if measured for unique-sequence nucleic acids in the genome and if expressed as mutations per cell division (or round of virus replication) per genome, is the same for all organisms (51). Thus, organisms with large genomes have fewer mutations per division per thousand base pairs than those with small genomes, but because they have larger genomes they will have the same overall number of mutations.

Regions of plant pathogen genomes evolve at different rates among different microbes, within microbes, and even at the individual base pair level. Viruses have different patterns of evolution (75), and some RNA viruses, such as influenza virus, evolve rapidly, while others, such as *Tobacco mosaic virus*, evolve slowly (60). In general, DNA viruses, particularly those with large genomes, evolve relatively slowly. For viruses, initiation of an infection with a genome that has been cloned in a bacterial plasmid rapidly results in the generation of a collection of genomic sequences whose diversity is characteristic of the virus and the host (162, 163). Forensic investigators should not limit characterizations to single cloned samples from suspect and crime scene viruses, since even fairly diverse sequences could be drawn from a single population of genomes. As noted earlier, TWSV isolates could be differentiated by analysis of five viral genes (178). Ideally, the population should be subjected to nucleotide sequencing directly, using analysis protocols that identify polymorphic positions.

For bacteria, common markers include the 16S rRNA, the spacer between the 16 and 23S rRNAs, and the *groE* and *recA* genes. Each has a slightly different rate of evolution, allowing coverage of several taxonomic levels. However, the rate of nucleotide substitutions is usually insufficient to establish that two bacteria with identical sequences for one of these genes had a common ancestor within a period consistent with forensic scenarios. Comparing the sequences of several genes, or of the whole genome, provides greater confidence. New developments in nucleic acid sequencing, such as a highly processive technique that employs amplification of DNA fragments in microspheres followed by pyrosequencing (113), allow rapid and cost-effective sequencing of entire microbial genomes. Finding one or more nucleotide differences between crime scene and reference sequences might be an indication that the suspect pathogen should be excluded as a source of the evidence microorganism. However, substitutions may have accumulated if many generations of propagation, or propagation in a selective environment, occurred since the two lines were separated. Separately propagated lines may also show dramatic changes in genome size (195).

Rates of molecular evolution of bacterial plant pathogens would be useful for forensic analyses, but the assumption that they have a molecular clock could be limited because selection for mutator strains (10) may occur after divergence of the suspect microbe from the common ancestor. Mutator strains constitute about 1% of natural populations (97), but their frequency (169) is increased during stationary phase (103). If one of the two bacteria being compared has had a high mutation rate since derivation from the parent, then the distance between the two bacterial isolates is inflated and can lead to inappropriate exclusion. On the other hand, estimates are that a thousand generations are needed to fix mutator strains (93) in a population (168).

Fungal retrotransposons provide a potential gene set with a level of variation sufficient for discrimination (62). Since several idiosyncrasies of their replication are documented, they are hot spots for the accumulation of nucleotide sequence changes. Different genes have different rates of evolution. Those rates are not constant at broad taxonomic levels, since the molecular clock for a particular protein-coding gene does not tick at the same rate in all lineages (6). Yet, at lower taxonomic levels, the assumption of a molecular clock has proven useful (15). Indeed, for fungi, the overall rates of molecular evolution have been judged indistinguishable for *Neurospora crassa* and *Saccharomyces cerevisiae* (14). In a series of four intraspecies comparisons based on whole bacterial genome sequences (*Chlamydia pneumoniae*, *Escherichia coli*, *Helicobacter pylori*, and *Neisseria meningitides*), essential genes were found to be more conserved than nonessential ones and duplicated genes (except in *C. pneumoniae*) had more differences than unique genes (83). Thus, duplicated genes (193) might be favored for microbial forensics, although distinguishing among nearly identical sequences may be a problem. Strain-specific genes tended to be uncharacterized ones.

Genomic processes other than nucleotide substitution may be occurring rapidly enough to assist the forensic investigator. Rates of deletions, inversions, and translocations per site, the expansion and contraction of regions of repeated sequences, the movement of mobile elements, the invasion of prophage genomes, and the acquisition or loss of plasmids all may provide useful clues. In a comparison of two *Chlamydia* species, the rates of deletions, inversions, and translocations per site were substantially less than the neutral substitution rate (42). Potentially more helpful are the expansion and contraction of regions of repeated sequences, the movement of mobile elements, the invasion of prophage genomes, and the acquisition or loss of plasmids. The utility of repeated sequences was examined for the fungus *Beauvaria bassiana* (39). Variation in the number of GA dinucleotide repeats occurred even within a pathotype or within a geographic region, suggesting a high level of discriminatory capacity. Expansion or contraction of a hexanucleotide sequence in a DNA repair gene is important for the generation of one kind of mutator strain of bacteria (167). In the *Saccharomyces* complex, the rate of genome re-

arrangement does not reflect phylogeny, since some of the most distant pairs had similar gene orders and some close pairs had numerous rearrangements caused by mobile elements (57). These events can be exploited to distinguish recently diverged microbes, but their quantitative treatment is not as straightforward as counting the nucleotide substitutions.

Importance of confidence to forensics. Identification of very close relatives of a crime scene microbe depends on the availability of comprehensive data sets covering the diversity of that species around the world and of validated ways to analyze them. The phylogenetic analysis of nucleotide sequences is a well tested and trusted method (184). Sequences of selected genes are known for most plant pathogens of concern but vary in the extent of coverage. For forensics, knowing the times of divergence of close neighbors within a model group could provide important clues. For a few model microbes we know how long ago some of the divergences in a phylogenetic tree occurred; thus, calculations can be carried out with some confidence on how long ago any pair of isolates of the model microbe diverged. That event might be used to calculate divergence dates. Often, the original isolate of a microbe will be propagated separately in multiple laboratories, leading to significant substrain differences. Although seldom are records of multiple laboratories and propagations sufficient to provide "chain of custody" evidence, the derived lines and the date of their split can still be useful to calculate a rate of divergence. Experience with separate propagation of the Ames strain of *Bacillus anthracis* failed to reveal differences among the laboratory propagates. Nevertheless, the anthrax case illustrates the use of the knowledge of divergence rates (98). In this case, the number of changes in the crime scene microbe relative to laboratory versions suggested separation by between 42 and 900 generations. The separation was not large enough to exclude the possibility that the crime microbe came from a number of laboratory stocks in the hypothesized time of separation. In another case in which divergence over time can be addressed more accurately, some SMV strains were capable of overcoming the resistance of soybean lines from populations of recombinant inbred lines having reassorted resistance genes (72). Directional selection due to divergence of SMV strain prevalence has been correlated with increased planting of resistant soybean cultivars (92).

The frequency of substitution or small deletion mutations depends on the fidelity of the DNA polymerase (or RNA polymerase), the amount of DNA (or RNA) damage encountered, and the mix and efficiency of DNA (or RNA) repair systems in the organism. While the error rates are likely fairly similar from one organism to another, the environments in which the organisms exist differ substantially as to how much damage their nucleic acid is likely to sustain from environmental stimuli (132). It is known that the mix of DNA repair systems differs among organisms. As a result, a rate of nucleotide sequence change calculated for one species may be quite different from that for a related species. Thus, although the study of model organisms that are relatively safe to work with can provide useful material for development of methods, ultimately the methods will need to be developed for the targeted agents.

Pathogen and Host Gene Expression and Protein Modification

Posttranslational protein modification. The complement of proteins that result from the transcription and translation of a pathogen's genes can provide a fingerprint of that organism that reflects environmental influences not revealed in the genome. Posttranslational protein modifications most commonly reported for plant pathogen proteins include glycosylation and phosphorylation. For *U. maydis*, the phosphorylation of some protein kinases is a critical component in regulation of genes involved in morphogenesis and pathogenesis (52, 66, 84, 118).

Some viral genomes, particularly in the *Potyviridae*, are translated as a single, large polyprotein that is subsequently cleaved into individual proteins by virus-encoded proteases (148, 182). Regulation of the process is presumably by interaction between the plant translation mechanisms and the viral genome. For other viruses, the coat protein is transcribed separately from a unique mRNA. The exact nature of the proteolytic processing that produces separated viral proteins may vary among strains of a virus, as is the case for SMV (111). For tospoviruses, viral glycoproteins are likely glycosylated prior to virion formation via the Golgi and associated membrane systems in the plant and/or the thrips vector (89, 123). Glycosylation sites of the glycoproteins have been examined, but as this protein modification is a host-driven process it is not likely to aid in forensic analysis.

Host-encoded products. In some cases, plant host cells have the ability to recognize and silence double-stranded forms of virus transcripts. In fact, viral infection often activates or inactivates transcription or translation of a number of host plant genes in both the susceptible and the resistant reactions (71). This reaction has been studied in the SMV system, in which variability in any of the viral or virus-specific host gene products could be targets for forensic exploration. Detection of such expression could be done easily with a microarray format. However, much more research is needed to determine whether such pattern-pathogen specificity would be reliable in differentiating pathogen isolates (36).

Pathogen-generated secreted products. The degree of variation in total protein profiles depends on the microbial taxon being studied. Overall, total protein profiles of the model pathogen *C. michiganensis* subsp. *sepedonicus* vary much less than those observed for other bacteria, even compared with subspecies of the same genus (30). Because of their involvement in host-pathogen interactions, secreted proteins, especially from bacteria, have come under greater scrutiny than an organism's total protein profile. Investigations of microbial secretomes, e.g., the set of proteins that are secreted by a microbe, have been especially informative in revealing conserved mechanisms of pathogenicity among bacterial plant pathogens. Pathogens produce several classes of secreted proteins, some of which function in virulence or plant defense (2).

Both genetic variation and environment influence secreted protein profiles. Profiles differ between virulent and avirulent strains of *C. michiganensis* subsp. *sepedonicus* and may be useful in identification (127). Several bacterial proteins, especially ones involved with host or cultivar specificity, are produced only in planta (141). The availability of complete genome sequences of several plant pathogens provides a resource for in

silico prediction of certain classes of secreted proteins. Targeted sequence analysis of these genes can become a means for strain differentiation and identification that circumvents difficulties associated with standardizing secreted protein profiles obtained from cell cultures. The challenge is being able to identify the entire set of secreted proteins, including those lacking obvious secretion sequence signals.

A phenotype of potential use with many plant pathogenic bacteria is the production, by some strains but not others, of a slimy capsule of extracellular polysaccharide, detectable as mucoid colonies (7). Bacterial gene expression in response to stress has been well studied in *P. syringae* pv. tomato DC3000, in which (as in many gram-negative bacteria) a GacS/GacA two-component regulatory system controls diverse phenotypes and processes, including pathogenicity, secretion systems, biofilm production, synthesis of secondary metabolites, survival, and extracellular polysaccharides (34). For forensics, one may be able to analyze such a polymorphic region of the genome to possibly generate a fingerprint. As mentioned previously, the EEL in the *hrp*-encoded PAI of *P. syringae* pv. tomato is a good candidate because it is divergent among pathovars (33, 48).

At least some members of the bacterial class *Mollicutes* (prokaryotes that lack cell walls: mycoplasmas, spiroplasmas, and phytoplasmas, among others) use yet another mechanism for introducing variability into their gene products. Phase variation, a form of phenotypic switching, is caused by frequent frameshift mutations, duplications or deletions of repetitive genome elements, and regulation of gene expression (183). As a result, surface membrane proteins, including adhesins, structural proteins, and transporter molecules, have variable sizes and structures. First described in human- and animal-pathogenic mycoplasmas, phase variation is thought to function to prevent recognition by the host immune system, thus facilitating pathogen establishment, and to provide additional opportunities for variation that could enhance niche adaptation. Phase variation might also be useful for discriminating among forensically interesting pathogen isolates. Evidence for differential antibody reactions among cells in a population and for repetitive elements in the genome of the plant pathogen *Spiroplasma citri* suggests that this group also may utilize phase variation (A. Wayadande et al. and U. Melcher et al., unpublished data). The phenomenon may confound microbe identification methods based on protein fingerprints but could be a tool for forensic investigations.

Standard Criteria for Isolate Discrimination and/or Matching

Levels of standardization and validation for methods currently used to identify plant pathogens are extremely variable. When standardization and validation are in place, it is usually for the purpose of certifying an agricultural commodity to be "pathogen free" for export/import markets or for seed development. One pathogen for which such measures are in place is the model bacterium *C. michiganensis* subsp. *sepedonicus*. Because ring rot disease, caused by this bacterium in potatoes, has been a serious quarantine issue and identification tests for pathogen detection have been available for many years (45), evaluations of the efficacy and reliability of some diagnostic tests exist (44, 46, 70). Comparisons of older, serological assays with PCR-based techniques are becoming available (120). The match criteria that finally determine a microbe's identity as *C. michiganensis* subsp. *sepedonicus* depend on the material being tested (79). The U.S. National Potato Council Seed Potato Certification Subcommittee is working to develop a set of match standards. Standards likely will be more stringent for certification of in vitro plantlets and greenhouse materials. Applying predetermined thresholds for each type of test and each type of plant material compensates for differences in the microbial background associated with the various materials.

There has been little incentive, for most plant pathogens, to invest limited resources in the development of tests that provide more information or a higher level of confidence than is necessary for identification. As in the case of *C. michiganensis* subsp. *sepedonicus*, the discriminatory tests for which the greatest numbers of pathogen strains or isolates have been tested are often older, more traditional approaches (44). For SMV, the reactions produced on differential host cultivars still provide the most definitive means of strain identification (4, 55). Molecular analyses (sequence analysis, RT-PCR, and restrictotyping) show promise but have not yet been applied to a sufficient number of virus isolates to be able to relate the results to those produced from reactions on host differentials or to provide a sense of the virus' variability in nature (49, 81, 92, 156).

At least one complete genome sequence is available for an increasing number of plant viruses. However, for viruses that have significant strain sequence diversity, such as PPV and SMV, a more in-depth characterization of full-sequence variants of many populations will be necessary to determine mutation rates and locations. To date, only 7 PPV and 10 SMV complete sequences are available in the GenBank database, limiting the use of sequence alignment for forensic analysis. However, partial sequences (of specific genes, such as the coat protein and the key strain-discriminating CI region of SMV) have been determined (49) for a number of isolates. The fact that plant viruses often mutate rapidly within their plant hosts means that uniquely matching a virus obtained from a plant sample to a virus from a suspect greenhouse culture would be difficult and likely will be based instead on a lineage similarity model.

The method most commonly used for distinguishing groups of fungi is the comparison of 18S rRNA genes. However, mating-type analysis is more useful for differentiating at the isolate level. Obtaining complete sequences of fungal pathogen genomes is an expensive and laborious prospect at present; thus, only a few genome sequences are currently available for plant pathogenic fungi (single strains of *Magnaporthe grisea*, the causal agent of rice blast, and *U. maydis* [http://www.broad.mit.edu/annotation/fungi/fgi/] are available, and genome projects for other species are in progress). Having at least low coverage of one or two additional strain sequences per fungal species would be very useful. Sequences of several *U. maydis b* mating-type alleles and fungal 18S rRNA sequences are available at the NCBI. Single nucleotide polymorphisms also would be useful for fungi.

New technologies could be extremely beneficial for achieving the degrees of reliability, sensitivity, and accuracy desired for microbial forensics. For bacteria, BIOLOG has developed new phenotype arrays, each providing information on about

2,000 phenotypic traits (e.g., surface structure; transport functions; catabolism of carbon, nitrogen, phosphorus, and sulfur; biosynthesis of small molecules; synthesis and function of macromolecules and cellular machinery; cellular respiratory functions; stress and repair functions) on sets of arrays (http://www.biolog.com/phenoMicro.html). Data are taken automatically several times per hour and are stored electronically to provide kinetic graphs, providing linkages to bioinformatics software. Biosensors, already developed for some human bacterial pathogens, are another area showing great promise; these have the capacity to detect multiple pathogens within a single sample. The ability to detect pathogens without labeling is an advantage provided by several new platforms, including surface plasmon resonance, acoustic, and calorimetric biosensors (40). Open reading frame-specific arrays or chips for strain identification would also be helpful.

Integrated Informatics and Data Analysis Strategy for Microbial Forensics

Microbial forensics requires the utilization of diverse types of data that are acquired through standard processes in distributed locations. Technologies for data production are evolving rapidly, especially with respect to instrumentation and techniques that produce high-resolution data about the molecular constituents of living cells (DNA, mRNA, proteins, and metabolites) that are used as pathogen signatures/fingerprints. Biological data, knowledge, and expertise are distributed throughout the country and the world among many different organizations. Both bioinformatics and computational biology have grown over the last 20 years, and diverse database systems and analytical tools have been developed and deployed. Some community resources, such as GenBank, have become key enablers of research on a global scale. The power of this distributed approach to development is that innovation has blossomed at various levels.

Not all data necessary for microbial forensics analysis resides in readily accessible databases. Architectures for databases to hold the unarchived data can be constructed according to established principles of database design. Filling those databases with the available and reliable data requires curation. Necessary initial and ongoing curation of data that requires specific biological knowledge, such as that involved in microbial forensics, is especially challenging because of the distributed nature of biological knowledge. The responsibility for such curation should be distributed among specialists. One model for such curation has been prototyped on a limited scale in the Pathogen Portal (PathPort) project (53). PathPort contains the Pathogen Information (PathInfo) resource, consisting of highly curated data sets, referenced from the scientific literature, from about 20 of the Department of Defense's 50 top-priority pathosystems (73). Automated text mining procedures need to be developed to assist and accelerate manual data curation efforts.

A question that frequently arises with infectious disease research is how to maintain appropriate security for the information contained in the databases. Such concerns should be balanced with the need for development of diagnostics, new treatments, and new disease-resistant varieties. Although it is certainly true that national biosecurity concerns merit highly stringent security measures, many do not realize that the intellectual property requirements associated with multimillion-dollar products of pharmaceutical and biotechnology companies have necessitated stringent informational security for many years. Web services (114) can provide security via models now being developed and implemented, such as WS-Security or OASIS WS Security TC. It is important to leverage community standards for implementation, and access can be based on individual users' security clearances.

Microbial forensics depends on the availability of reliable data, the development of rigorous data management, and the capability to use the most appropriate analyses. Data and analysis/visualization tools are available, requiring information system architectures that leverage old techniques as well as enable rapid deployment of new ones. The challenge is that there have been relatively few concerted efforts to standardize data formats, thus hindering efforts to integrate disparate data types from diverse data sources. Further synthesis in biology largely depends on the capability to access and jointly analyze disparate data. This is especially true for microbial forensics, in which data from many types of organisms (pathogens and their hosts) originating from diverse environments (e.g., intracellular to field agriculture) must be considered. Interestingly, as the explosion of types and volume of data occurs, there is an ongoing change in software architectures that support data integration and interoperation, with a gradual change from a client-server to a web services approach to data analysis. The latter approach is enabled because of agreement on standards across a very broad range of hardware and software organizations. For concerns related to biosecurity, biosurveillance and bioforensics, this technological advance is enabling because information systems interoperation is needed to support collaboration across organizations and real-time information access and analysis. There is a danger that standards may be fragmented if diverse, noninteracting groups build competing extensible markup languages (XMLs) covering essentially the same data. Avoiding such duplication will require diligence, incentives from funding agencies, and requirements for machine-readable interfaces to major resources that are built with federal funding. Eventually, the achievement of interoperability among systems will be its own incentive, because those not conforming will not be competitive or generally useful.

Effective responses to natural, accidental, or intentional disease outbreaks will require that information be easily accessed in real time or near-real time. Flexible, decentralized, modular information system architectures able to adapt to evolving requirements, and available on the Internet, are needed. A client-side connecting application, ToolBus, facilitates single-interface access to diverse community resources via web services (53). Web services use the agreed-upon standards for exchange of data.

A number of efforts are now using the PathPort cyberinfrastructure. For example, PathPort is linked with other systems to provide the Bioinformatics and Genomics Research Core for the Mid-Atlantic Regional Center for Biodefense and Emerging Infectious Diseases. In this large, multi-institutional program, part of a national network funded by the National Institute of Allergy and Infectious Diseases (NIAID) in 2005, the main objective is to develop countermeasures for infectious agents on NIAID category A and B priority lists. A range of other activities, from real-time video conferencing to interactive tools supporting document preparation, discussion of data,

and presentations, etc., are supported with the goal of a vibrant, functional cyberinfrastructure for infectious disease research. As different agencies and scientists working on different aspects of infectious diseases use and help evolve the cyberinfrastructure, it will be possible to jointly analyze data sets that were developed with specific goals in mind but that can be useful to other goals. Other examples include the Pathosystems Resource Integration Center, PATRIC, one of eight Bioinformatics Resource Centers funded by NIAID, and the administrative center for proteomics biodefense, also an NIAID program. All of these networks develop data that can be used for vaccines, therapeutics, and diagnostics, and therefore there is a great need to make the systems interoperable by design. The same applies to forensics.

In the business setting, web services are succeeding the client-server architectures of the 1990s as the main driver for providing platforms for integration and interoperation, as well as for real-time acquisition and analysis of data sets. Just-in-time manufacturing is built on this type of platform. The need in the life sciences community has not been fully realized, but a cyberinfrastructure that will allow increased interoperability and new forms of organizational collaboration, beyond the current model based on the Internet and web browsers alone, is needed. Fortunately, the developments in the business community can be leveraged for the life sciences. In particular, an area that will increasingly need interoperability and real-time analysis is infectious disease research and development. Microbial forensics is only one of many components crucial to the growing biosecurity needs of the nation and the world.

BUILDING PLANT PATHOGEN FORENSIC CAPABILITY: NEAR- AND LONG-TERM STRATEGIES

The analysis presented in this study, developed by a team consisting of plant pathologists, academicians, a USDA scientist, and members of the law enforcement and intelligence community, is a critical starting point in the recognition of a national need in plant pathogen forensics, the assessment of available tools and resources, and the identification of gaps and needs. But it is only a first step. The next steps should continue to be made in the framework of a partnership between these and other entities so that the outcomes are truly effective and responsive to national needs.

Forensics-relevant databases, tools and technologies, culture collections, and scientist expertise cross disciplinary boundaries. Many of the concerns and strategies that exist for human and animal health and security are relevant also to plant security, needing only to be adapted for application in a different type of setting or to different specific microbes.

Recent and Current Initiatives

Traditionally, as well as currently, forensic issues have been addressed by the Federal Bureau of Investigation (FBI), which has established a Scientific Working Group on Microbial Genomics and Forensics (SWGMGF), and the Central Intelligence Agency. The more recently established Department of Homeland Security's National Biodefense Analysis and Countermeasures Center (NBACC) will integrate all of the nation's efforts for homeland security, drawing on resources from public health, veterinary science, plant pathology, law enforcement, and national security. A comprehensive microbial forensics program, part of the NBACC mission, is addressed by the National Bioforensic Analysis Center.

Addressing issues of plant biosecurity, the USDA's APHIS, Agricultural Research Service (ARS), and Cooperative State Research, Education and Extension Service (CSREES) continue to function in their respective roles (regulatory; in-house research; and the land grant university system of research, teaching, and extension, respectively). They are augmented by CSREES's recently established National Plant Diagnostic Network (NPDN), a distributed set of plant pathogen and pest diagnostic laboratories that was built from an existing system of diagnostic laboratories at land grant universities in each state (170, 171). Each state laboratory reports new or unusual occurrences to a regional hub laboratory, where data are entered into a national database for analysis and action. This system has great promise, but much is still in development and the NPDN does not presently address forensics issues. Additional national coordination with a reliable, continuing funding base is needed to ensure the long-term success of the NPDN.

In 2004, President George W. Bush issued Homeland Security Presidential Directive 9 (HSPD-9), which called for the establishment of a national policy to defend the nation's agriculture and food system against terrorist attacks, major disasters, and other emergencies, because such events could have catastrophic health and economic effects. A key component of HSPD-9 was the development of a National Plant Disease Recovery System (NPDRS) that would ensure the ability to recover from any intentional or unintentional outbreaks of a high-consequence plant disease. HSPD-9 directed the Secretary of Agriculture to accelerate and expand agricultural biosecurity efforts in cooperation with other federal agencies and entities. The NPDRS, now administered from the USDA's Office of Pest Management Policy, is clearly a critical component of our national plant security system, but its priorities currently do not include forensic issues.

Although all of these agencies are important to the nation's overall preparedness for agricultural security, their missions and roles do not directly address the issues of plant pathogen forensics. A new initiative is called for, with a primary focus to develop this new discipline by prioritizing needs, developing tools, and training personnel, always in cooperation and communication with related biosecurity initiatives. Scientific societies are available to assist in this effort by playing an important role in facilitating multidirectional communication, providing venues for training and discussion, and serving as clearinghouses for scientific exchange in the relevant science and practice. For example, the American Phytopathological Society has established a Working Group on Plant Pathogen Forensics, whose objectives include informing plant pathologists about forensic issues via conference symposia and publications and contributing to the national forensic community via a representative on the FBI's SWGMGF.

Gaps Assessment and Recommendations

The development of a strong initiative in plant pathogen forensics is hampered by gaps in several critical areas, including the dearth of key personnel trained in applied plant pa-

thology and in forensic science, the scarcity of laboratory facilities containing the required biosafety and biocontainment equipment, and the lack of targeted grant funds and other resources for both seasoned and creative young scientists to carry out the research needed to support this initiative.

Gaps in personnel. More knowledgeable field personnel are needed to achieve an effective national plant security system and, in the case of a criminal event, to identify timely response steps consistent with the need for forensic investigation. The Cooperative Extension Service in the United States, which is supported by federal, state, and county funding, has long served as a critical link between the land grant university (now NPDN) diagnostic labs and the agricultural producers. However, continuing financial limitations over a period of years have led to significant understaffing in extension. Concomitant cutbacks in academic programs and an increasing emphasis on fundamental rather than field-directed research have produced a serious deficit in the number of graduate students being trained for field applications. In addition, valuable and undertapped human resources exist in our crop consultants, agricultural industry representatives, and amateur groups such as Master Gardeners. Funds targeted to applied plant pathology graduate programs and training programs for other key responders are critically needed to prevent this lack of personnel from becoming one of the most serious gaps in our crop biosecurity efforts.

Gaps in infrastructure. High-level containment (BL-3 and BL-4) facilities, required for handling high-risk exotic plant pathogens, are very expensive to build and to secure. Thus, the number of locations able to support research on, or activities related to, these select agents is very small. A coordinated plan for increasing our physical capacity for containment activities is crucial. It might include the placement of new laboratories and/or the upgrading of existing facilities in key locations or in locations where there would be no consequence if a high-risk pathogen were accidentally released, such as in geographical regions where no plant host is present or the pathogen could not survive outside the facility.

Gaps in research and technology. As the nascent discipline of plant pathogen forensics develops, standard crime scene processing and evidence handling protocols must be validated and adapted to plant pathogen forensics applications. It may be appropriate to develop new technologies specific for crime scenes involving crops, forests, nurseries, orchards, or rangelands.

Building Plant Pathogen Forensic Capability

The discipline of plant pathogen forensics, currently in its infancy, will require intellectual and financial investment to develop an effective level of robustness and utility. The existing physical and human infrastructure in the United States for plant pathogen detection, diagnosis, forensics, and research has become strained as officials and the scientific community worked to develop and implement new programs to address crop biosecurity issues. There are significant gaps in research, technology, education, and training. Current efforts to establish plant pathogen forensics programs compete for limited resources with more-established human and animal biosecurity programs. However, purpose-driven action and targeted financial resources are essential to build the necessary programs, personnel, and infrastructure.

The next stage in building capacity in plant pathogen forensics should be the development of a detailed strategic plan, to include specific and prioritized issues and needs as defined and identified by forensic specialists working together with agricultural leaders. A thorough systems analysis of the existing science is needed to further define current and future threats and risks to the U.S. agricultural enterprise from the deliberate use of plant pathogens. The plan also should include a time line for the research, development, testing, validation, and implementation of methods, technologies, and practices that will establish plant pathogen forensics and incorporate it into the national agricultural biodefense "toolbox."

Various initiatives on the part of existing U.S. agencies to redirect efforts and to initiate new programs to enhance national security of our crops, rangelands, and forests have strengthened national agricultural security. However, the establishment of a single federal coordinating body, a National Center for Plant Biosecurity (NCPB), for plant pathogens and crop health could provide focus, coordination, and strategic planning for such efforts. The NCPB, as described in a proposal by the American Phytopathological Society and coauthored by a number of other scientific societies (http://www.apsnet.org/members/ppb/PDFs/CenterProposal_Final.pdf), would be able to build on, enhance, and provide leadership and coordination of national efforts for documenting, monitoring, and protecting crops, forests, and rangelands against new or emerging plant diseases.

Because most high-risk pathogens (by definition) are not now present in the United States, much of the scientific research on them is done overseas. Furthermore, because intentional introduction of such pathogens into the United States could be an international incident, forensic investigation of such events will stretch across national borders. Plant pathogens do not recognize political boundaries; the introduction of a plant pathogen into agricultural areas in a country adjoining the United States often results in the eventual movement of the pathogen into the United States. Thus, no national security plan can be optimally effective on its own. Plans developed, adopted, and monitored at a global level will be most effective. The National Research Council (NRC) has called for harmonized international oversight of biosecurity efforts (124, 125). Whether this is sought by a meeting of nations (an International Forum on Biological Security, sponsored by governmental and nongovernmental entities, was proposed by the NRC) or by a series of more focused interactions, the goal would be to strengthen research and operational links for the enhancement of global security.

CONCLUSIONS

Strategies for securing our plant resources (crops, rangelands, and forests) must address both prevention and preparedness (5). Capabilities needed to respond to an attack, should one occur, must include microbial forensic technology and practice to allow determination of the source of the pathogen and to provide evidence needed to attribute the act to those responsible. This component should include strategies for (i) assuring high stringency (validation, confidence, statis-

tical significance, consistency), (ii) tracing pathogen origin and movement, (iii) discerning the timing and site of the initial introduction, (iv) identification of the perpetrators, (v) collection of evidence for criminal attribution, and (vi) establishment of links to the law enforcement and security communities. New investments in research, infrastructure, personnel, training, and strategic planning will be critical in meeting the needs of our overall national security programs.

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